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(54) Title: **HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES**

(57) Abstract: Double-labeled protein biomolecular substrates and methods for the homogenous assay of processes by which biomolecules are covalently modified are described. The methods of the present invention utilize biomolecular substrates labeled at two positions with two fluorescent dyes or with a fluorescent dye and a nonfluorescent dye. The two labeling dyes of the unmodified biomolecular substrates stack, thereby quenching the substrate's fluorescence. Upon covalent modification of the double-labeled substrate, however, the intramolecularly stacked dyes dissociate and the fluorescence of the phosphorylated substrate changes markedly. Methods utilizing the double-labeled substrates of the present invention do not require physical separation of modified and unmodified substrate molecules, nor do they require other special reagents or radioactive materials. Methods for preparing and characterizing the substrates used in the assay procedure are described, as are methods utilizing the substrates of the present invention for high-throughput screening, for monitoring intracellular processes of covalent biomolecular modification in living cells, for diagnostic and therapeutic applications for diseases involving dysfunctional processes of covalent biomolecular modification, and for discovering novel enzymatic substrates.

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HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES

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PRIORITY CLAIM

This application claims the benefit of the filing date of United States Provisional Patent Application Serial Number 60/145,755, filed July 27, 1999, for "HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES".

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TECHNICAL FIELD

The invention relates to methods useful for the homogenous assay of covalent modifications to biomolecules which utilize double-labeled biomolecular substrates. Also described are methods of preparing and characterizing the double-labeled biomolecular substrates and methods of using the inventive assay for high-throughput screening, for diagnostic and therapeutic applications, and for discovering substrates of novel enzymes.

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BACKGROUND

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The healthy development and function of eukaryotic organisms depends upon the proper regulation of structural modifications of various biomolecules (the phrases "covalent modification" and "structural modification" are used interchangeably herein). It is believed that virtually all intracellular biochemical processes in eukaryotes are regulated in some fashion by the covalent modification of biomolecules, such as proteins or peptides. However, where an intracellular process responsible for the covalent modification of a particular type of biomolecule is somehow dysfunctional, several disease states can result.

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For example, protein kinases represent one of the largest superfamilies of enzymes in eukaryotic organisms, with an estimated 1-3% of the human genome coding for various protein kinases. Protein kinases catalyze the transfer of phosphate from ATP to specific amino acids in proteins, and phosphorylation of proteins is known to be the most widespread mechanism for reversible covalent modification of protein structure and function. The dysfunctional regulation of protein phosphorylation is

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believed to result in several diseases, such as, for example, diabetes, cancer, and many forms of heart disease.

As can be appreciated, the ability to assay the activity of the various intracellular processes responsible for the covalent modification of particular biomolecules is essential in order to gain an understanding of the potential roles such processes play in normal cells and various disease states. Assay techniques which detect and quantify various types of covalent modifications of particular biomolecules would also facilitate the development of diagnostic and therapeutic technologies relating to disease states resulting from dysfunctional modification processes. An assay technique ideally suited for these purposes would be sensitive and continuous, would allow both *in vitro* and *in vivo* assays, would be efficient and economical, and would enable high-density, high-throughput screening.

A variety of methods are currently used to assay the covalent modification of biomolecules. Most of these methods, however, are relatively inefficient or uneconomical in that they require the use of radioactive labels, multi-component assay systems, and/or multi-step procedures. Moreover, most existing methods of assaying the structural modification of biomolecules are discontinuous and, therefore, necessitate the sampling of the reaction at specific times in order to determine enzymatic activity.

Unlike most known methods, U.S. Patents 5,776,720 to Jaffe et al. (July 7, 1998), 5,770,691 to Fields et al. (June 23, 1998), 5,763,181 to Han et al. (June 9, 1998), 5,733,719 to Jaffe et al. (March 31, 1998), and 5,698,411 to Lucas et al. (December 16, 1997) teach various methods for assaying enzyme-mediated cleavage of biomolecules, such as peptides or nucleic acids, which do not require radioactive labels and which are continuous and homogenous. However, the usefulness of even these methods is limited to the assay of enzyme-mediated cleavage reactions, and such reactions constitute only one subset of the many processes by which biomolecules are structurally modified within the cell.

Homogenous assay methods which detect the presence of antibodies, nucleic acids, or protein kinase activity through the use of fluorogenic tracer molecules are also known. For example, PCT International Publication No. WO/03429 ("the WO/03429 publication") teaches homogenous assay techniques which utilize fluorogenic tracer molecules that exhibit a change in fluorescence upon association with their target

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antibody or nucleic acid sequence. Though the WO/03429 publication provides no solution to the challenge of developing methods or compositions for monitoring or detecting covalent biomolecular modifications, a protein kinase assay technique making use of the technology disclosed in the WO/03429 application was recently reported in an article authored by Geoghegan et al. ("Geoghegan et al.") (*See*, Geoghegan et al., *Bioconjugate Chemistry*, 11:71-77 (2000)).

The assay technique disclosed in Geoghegan et al. is relatively complicated and expensive, however, and the technique unsuitable for *in vivo* application. The assay technique disclosed in Geoghegan et al. uses double-labeled tyrosine kinase substrate peptides and a polypeptide corresponding to an SH2 domain. The SH2 domain binds the double-labeled tyrosine kinase substrate peptides when those substrate peptides are phosphorylated, and binding of the SH2 domain to the phosphorylated double-labeled tyrosine kinase substrate peptides causes the two labels included in each of the substrate peptides to dissociate, resulting in a change in fluorescence. Thus, the assay technique disclosed in Geoghegan et al. requires the interaction of two expensive components (*i.e.*, the interaction of double-labeled tyrosine kinase substrate peptides with SH2 domain peptides), resulting in a technique that is relatively complex and expensive. Moreover, due to its use of a multi-component system, the assay technique of Geoghegan et al. is not suitable for performing *in vivo* assays.

Homogenous fluorescent protein kinase assay methods utilizing various Green Fluorescent Protein ("GFP") molecules have also been recently reported. For instance, an article written by Nagai et al. teaches a method for assaying protein kinase activity using a Kinase-Inducible Domain construct containing two GFP groups (*See*, Nagai et al., *Nature Biotechnology*, 18:313-316 (2000)). The assay method taught by Nagai et al., however, depends upon phosphorylation-dependent changes in the fluorescence resonance energy transfer ("FRET") among the two GFP groups to detect kinase activity, and because such changes in FRET are small, the assay technique of Nagai et al. does not provide a homogenous assay having a desired level of sensitivity. In addition, U.S. Patent 5,912,137 ("the '137 Patent") teaches a protein kinase assay utilizing modified GFP molecules as assay substrates. However, because the assays taught in the '137 patent can only be carried out using modified GFP substrates, the potential applications of such assays are limited.

Because of the deficiencies existing in the known assay methods, it would be an improvement in the art to develop a homogenous assay method which not only enables the continuous, real-time assay of covalent biomolecular modifications, such as phosphorylation, sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, and biotinylation, but which also provides sensitive and economical assays that are simple to carry out *in vitro* and *in vivo* and are adaptable to a wide variety of applications.

DISCLOSURE OF INVENTION

The present invention includes substrates and methods useful for the assay of covalent modification of biomolecules. In contrast to assay methods already in use, due to the nature of the double-labeled molecular substrates described herein, the assay methods of the present invention are sensitive and homogeneous and do not require the use of radioisotopes. The assay methods herein disclosed are also relatively simple and economical, adaptable to a wide variety of applications, easily used *in vitro* and in living cells, and allow continuous, real-time monitoring of structural modifications to biomolecules. Moreover, the assay methods of the present invention are useful for detecting and quantifying a wide range of covalent biomolecular modifications which do not result in the cleavage of the biomolecule. Thus, the present invention offers significant advantages in terms of simplicity, efficiency, and scope when compared to presently used methods for detecting covalent biomolecular modifications.

The substrates of the present invention are double-labeled biomolecular substrates (the phrases "double-labeled biomolecular substrate" and "double-labeled substrate" are used interchangeably herein). The double-labeled substrates of the present invention include a core molecular backbone covalently labeled at two positions with a first fluorescent dye and a second fluorescent dye or with a first fluorescent dye and a second non-fluorescent dye (for convenience, the term "dye" is used herein to describe a chromophoric or fluorophoric moiety). The core molecular backbone of a double-labeled substrate according to the present invention may include a protein or peptide sequence, a nucleotide sequence, a sugar, a lipid, a receptor, or a biopolymer. As used in the context of the present invention, the term "biopolymer" includes any molecule that is a covalent combination of amino acids, nucleic acids, sugars, lipids, or

other small molecules of biological origin. The core molecular backbone may also include a substrate determinant specific for a particular process of covalent biomolecular modification. Furthermore, each double-labeled substrate of the present invention is constructed and labeled so that when the double-labeled substrate is in its unmodified state, the first and second labeling dyes associate, or stack, to form an intramolecular dimer. When the first and second labeling dyes form an intramolecular dimer, the fluorescence of one or both of the dyes is quenched, thereby quenching the fluorescence of the double-labeled substrate. Upon catalytic or non-catalytic covalent modification of the double-labeled substrate, however, the first and second labeling dyes dissociate, and the fluorescence of the covalently modified double-labeled substrate changes markedly. Therefore, because only the modified double-labeled substrate exhibits a change in fluorescence, no need exists to separate the modified double-labeled substrate from the unmodified double-labeled substrate in order to accurately assay the extent to which an amount of double-labeled substrate has been covalently modified. As a result, the invention enables homogenous and continuous assay methods which are simple and economical, and which may be employed both *in vitro* and in living cells.

It should be noted that the quenching phenomenon underlying the substrates and fluorescence assay methods of the present invention is not fluorescence resonance energy transfer ("FRET"). Unlike FRET, the quenching phenomenon underlying the substrates and fluorescence assay methods of the present invention involve ground state interaction of two dyes. Advantageously, because the quenching phenomenon described herein involves ground state interactions which result in changes in the absorbance spectra of the two dyes included in a double-labeled substrate according to the present invention, double-labeled substrates of the present invention may be used for homogenous absorbance-based assays detecting various types of structural modifications of biomolecules through modification-dependent changes in the absorbance spectra of the double-labeled substrates.

The assay methods of the present invention are versatile. Because the core molecular backbone of double-labeled substrates of the present invention can be constructed to include substrate determinants for a wide range of intracellular processes, the assay methods of the present invention are applicable to a broad range of

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covalent biomolecular modifications. For example, the core molecular backbone of a double-labeled substrate may include a protein kinase substrate determinant. Such a double-labeled substrate could then be used to assay the activity of a particular protein kinase or a particular class of protein kinases. However, this is but one example.

- 5 Double-labeled substrates of the present invention could be constructed in order to assay numerous other modification reactions, such as sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, and biotinylation, by which biomolecules are structurally modified.

- Various other methods are also included within the scope of the present
10 invention. For example, methods of producing the assays of the present invention and of producing and characterizing the double-labeled biomolecular substrates of the present invention are described herein. Also described herein are methods for using the inventive assay procedure in high-throughput screening, methods for monitoring the activity of the intracellular processes by which biomolecules are covalently modified,
15 methods for diagnostic and therapeutic applications of the inventive substrates and assay procedures, and methods for discovering substrates for novel enzymes involved in the covalent modification of particular biomolecules.

BRIEF DESCRIPTION OF DRAWINGS

- 20 FIG. 1 provides a schematic illustration of a first embodiment of a double-labeled biomolecular substrate of the present invention in an unmodified state.

FIG. 2 is a schematic illustration of a first embodiment of a double-labeled biomolecular substrate of the present invention in a structurally modified state.

- FIG. 3 schematically depicts the modification-dependent transition between
25 intramolecular dimer and intramolecular monomer states of a double-labeled biomolecular substrate according to the present invention.

FIG. 4 is a schematic illustration of a second embodiment of a double-labeled biomolecular substrate of the present invention in a structurally modified state.

- FIG. 5 illustrates the absorbance spectrum of double-labeled substrate including
30 a fluorescein and a rhodamine label before and after phosphorylation by PKA.

FIG. 6 illustrates the fluorescent emission of the fluorescein label of the double-labeled substrate of FIG. 5 before and after phosphorylation by PKA.

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FIG. 7 illustrates the fluorescent emission of the rhodamine label of the double-labeled substrate of FIG. 5 before and after phosphorylation by PKA.

FIG. 8 illustrates an absorbance spectrum of double-labeled substrate including two rhodamine labels before and after phosphorylation by PKA.

5 FIG. 9 is a chart of the fluorescence the rhodamine labels of the double-labeled substrate of FIG. 8 before and after phosphorylation by PKA.

FIG. 10. schematically illustrates a double-labeled substrate including a covalently attached enhancer, the double-labeled substrate transitioning between an unphosphorylated and a phosphorylated state upon phosphorylation by PKA.

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BEST MODES FOR CARRYING OUT THE INVENTION

A first embodiment of a double-labeled biomolecular substrate according to the present invention is schematically illustrated in its unmodified state in FIG. 1. The double-labeled substrate 10 includes a core molecular backbone 20 containing a
15 substrate determinant 30 which facilitates the modification of the double-labeled substrate, a fluorescent dye 40, and a quenching dye 50. As is illustrated in FIG. 1, the double-labeled substrate 10 is constructed so that, in the substrate's unmodified state, the fluorescent dye 40 and the quenching dye 50 associate, or stack, to form an intramolecular dye dimer 55. The formation of an intramolecular dye dimer 55 results
20 in the quenching of the fluorescent dye 40 by the quenching dye 50. Thus, in its unmodified state, the fluorescence of the double-labeled substrate 10 is markedly reduced.

FIG. 2 provides a schematic illustration of the double-labeled substrate 10 of FIG. 1 after the double-labeled substrate 10 has been structurally modified by, for
25 example, a protein kinase. As is illustrated in FIG. 2, the protein kinase adds a phosphate group 60 to an amino acid residue of the substrate determinant 30 included in the core molecular backbone 20 of the double-labeled substrate 10 (protein kinases most commonly phosphorylate hydroxyl amino acids, such as serine, threonine, and tyrosine). Phosphorylation of the double-labeled substrate 10 results in the dissociation
30 of the fluorescent dye 40 and the quenching dye 50 and, thus, the dissociation of the intramolecular dye dimer 55, which, in turn, results in a marked increase in the fluorescence of the double-labeled substrate because the quenching dye 50 no longer

quenches the fluorescence of the fluorescent dye 40. The double-labeled substrate 10 only exhibits a change in fluorescence upon covalent modification, in this case phosphorylation. Thus, the extent to which an amount of double-labeled substrate of the present invention is structurally modified can be continually assayed without
5 separation steps merely by quantifying various changes in substrate fluorescence.

FIG. 3 schematically illustrates the modification dependent transition between intramolecular dimer and intramolecular monomer of the two dyes 80, 90 of a double-labeled substrate 70 of the present invention using a peptide based on the Kinase-Inducible Domain (KID) of the Cyclic AMP Response Element Binding Protein
10 (CREB) and Protein Kinase A ("PKA") as an illustrative system. CREB is a well-known substrate for PKA and other protein kinases, and FIG. 3 illustrates a double-labeled substrate 70 of the present invention which includes the KID sequence of CREB within the core molecular backbone 75, and two dyes 80, 90 conjugated to the KID sequence. In the double-labeled substrate's 70 unphosphorylated state, the two
15 dyes 80, 90 form an intramolecular dye dimer 95 and the fluorescence of the fluorescent dye 80 is quenched. When the double-labeled substrate is reacted with PKA however, a phosphate group, or "P" 100, is introduced in the double-labeled substrate 70, and the intramolecular dye dimer 95 dissociates, resulting in an increase in the fluorescence of the fluorescent dye 80.

20 Though the double-labeled substrate of the present invention and the modification-dependent transition of double-labeled substrates of the present invention have thus far been described in the context of protein kinase activity, such descriptions are illustrative only and do not limit the scope of the present invention. The double-labeled substrates of the present invention are useful for assaying a broad range of
25 structural modifications to various biomolecules and can be specifically constructed for the assay of numerous processes of covalent biomolecular modification. The core molecular backbone of double-labeled substrates of the present invention may be constructed to include protein or peptide sequences, nucleotide sequences, sugars, lipids, receptor molecules, biopolymers, or virtually any other biomolecule which may
30 serve as a substrate in one or more intracellular processes of covalent biomolecular modification. Thus, double-labeled substrates of the present invention can be constructed for the assay of numerous catalytic and non-catalytic processes of covalent

biomolecular modification. By way of example, double-labeled substrates according to the present invention could be constructed to exhibit a change in fluorescence upon sulfation, glycation, glycosylation, carboxylation, myristoylation, farnesylation, ubiquitination, biotinylation, or other modification reactions.

5 Although not intending to be bound by a particular theory of the invention, the following explanations might explain the excellent results of the invention. For instance, the dissociation of intramolecular dye dimer formed by the two dyes could be the result of one or more different mechanisms. The simplest mechanism of intramolecular dye dimer dissociation would be through steric and/or electrostatic
10 effects resulting from the introduction of a functional group into the double-labeled substrate in close proximity to the intramolecular dye dimer. Another possible mechanism for the dissociation of the intramolecular dye dimer is through a modification-dependent conformational change in the double-labeled substrate. Yet another possible mechanism for the dissociation of the intramolecular dye dimer is
15 through a conformational change in the double-labeled substrate brought about by the modification-dependent binding of a second molecule to the double-labeled substrate. These second molecules could be considered as fluorescence enhancers. For example, where the double-labeled substrate is modified by the addition of a phosphate group to a tyrosyl residue in a peptide substrate, the enhancer molecule might be a
20 phosphotyrosine-specific antibody or a SH2-domain-containing protein with a high affinity for phosphotyrosine. As is illustrated in FIG. 10, an enhancer molecule 200 could be covalently combined with the double-labeled substrate 10 to form a single chimeric molecule.

 A second embodiment of the double-labeled substrate of the present invention is
25 schematically illustrated in FIG. 4. The double-labeled substrate 110 of FIG. 4 is illustrated in its modified state for ease of description and includes a core molecular backbone 120, a substrate determinant 125 within the core molecular backbone 120, a first spacer segment 130 and a second spacer segment 140. The first spacer segment 130 is included at a first terminus 135 of the core molecular backbone 120, and
30 the second spacer segment 140 is included at a second terminus 145 of the core molecular backbone 120. The first or second spacer segments 130, 140 may be included in the double-labeled substrate 110 to provide a region of flexibility between

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the core molecular backbone 120 and one or both of the dyes 150, 160 also included in the double-labeled substrate 110. By providing a region or regions of flexibility between the core molecular backbone 120 and one or both the dyes 150, 160, the spacer segments 130, 140 may ease the formation of an intramolecular dye dimer, and thus
5 facilitate more complete quenching of double-labeled substrate 110, resulting in a more sensitive assay. The first or second spacer segments 130, 140 may also be included in the double-labeled substrate 110 in order to facilitate the use of a particular dye which cannot be conjugated to one of the amino acid residues included in the core peptide sequence 120.

10 The embodiment illustrated in FIG. 4 highlights that the exact construction of double-labeled biomolecular substrates of this invention will vary. The construction of a double-labeled substrate may vary not only by including one or more spacer segments which may facilitate a more sensitive assay or enable the use of different dyes, but the construction may vary to utilize biomolecular substrates corresponding to different
15 processes of covalent biomolecular modification. For example, within the family of protein kinases, double-labeled substrates of the present invention could be constructed using core molecular backbones which include, among others, the following amino acid sequences: Arg-Arg-Arg-Val-Thr-Ser-Ala-Ala-Arg-Arg-Ser (SEQ. ID. NO.: 9), a substrate peptide for Protein Kinase A and Protein Kinase C (*See, e.g.*, PCT
20 International Application WO Patent Document 98/09169); Phe-Arg-Arg-Leu-Ser-Ile-Ser-Thr (SEQ. ID. NO.: 1) and Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser (SEQ. ID. NO.: 2), substrate peptides for Ca²⁺/calmodulin-dependent protein kinase II (*See, e.g.*, PCT International Application WO Patent Document 98/09169; Pearson et al., *Journal of Biological Chemistry*, 260(27), 14471-76 (1985)); Phe-Leu-Thr-Glu-Tyr-Val-Ala-
25 Thr-Arg-Trp-Tyr-Arg-Ala-Pro-Glu (SEQ. ID. NO.: 3), a substrate peptide for mitogen-activated protein kinase kinase (*See*, Rossomondo et al., *Proceedings of the National Academy of Science USA*, 89, 5221-25 (June 1992)); or Arg-Arg-Asp-Ile-Tyr-Glu-Thr-Asp-Tyr-Tyr-Arg-Lys (SEQ. ID. NO.: 4), a substrate peptide for insulin receptor protein-tyrosine kinase (*See*, Dickens et al., *Biochemical and Biophysical Research
30 Communications*, 174(2), 772-84 (1991)). These examples, however, illustrate only a few of the potential substrate determinants which may be included in double-labeled substrates of the present invention. These examples do not reflect the myriad of other

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20 International Application WO Patent Document 98/09169); Phe-Arg-Arg-Leu-Ser-Ile-Ser-Thr (SEQ. ID. NO.: 1) and Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser (SEQ. ID. NO.: 2), substrate peptides for Ca^{2+} /calmodulin-dependent protein kinase II (*See, e.g.,* PCT International Application WO Patent Document 98/09169; Pearson et al., *Journal of Biological Chemistry*, 260(27), 14471-76 (1985)); Phe-Leu-Thr-Glu-Tyr-Val-Ala-
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30 protein-tyrosine kinase (*See, Dickens et al., Biochemical and Biophysical Research Communications*, 174(2), 772-84 (1991)). These examples, however, illustrate only a few of the potential substrate determinants which may be included in double-labeled substrates of the present invention. These examples do not reflect the myriad of other

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biomolecular substrates which may be included in the core molecular backbone of double-labeled substrates of the present invention. Again, the double-labeled substrates of the present invention have broad application and can be tailor-made for use with any one of many enzymatically catalyzed or non-catalyzed intracellular processes by which biomolecules are covalently modified.

It is also possible to create double-labeled substrates according to the present invention using a variety of dyes and combinations of dyes. For example, the dyes may be conventional fluorescent dyes, such as fluorescein, rhodamine, cyanine, Oregon Green, Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin, naphthalene, pyridyloxazole, anthracene, fluorescamine, acridine, benzofuran, anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole, carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimane, or phenanthroline, or the dyes may be non-conventional fluorescent dyes, such as a Yellow Fluorescent Protein (YFP) or a Green Fluorescent Protein (GFP) (obtainable from CLONTECH Laboratories, 1020 East Meadow Circle, Palo Alto, CA 94303). In addition, the publication entitled "*Handbook of Fluorescent Probes and Research Chemicals*," by Richard P. Haugland, which serves as a catalog for Molecular Probes, Inc., of Eugene, Oregon, sets forth additional fluorescent dyes that may be used in constructing the double-labeled substrates of the present invention. However, the dyes listed here, as well as those described within in the *Handbook of Fluorescent Probes and Research Chemicals*, are provided for illustrative purposes only and do not comprise a comprehensive list of the dyes usable in the context of the present invention.

A double-labeled substrate according to the present invention may include a non-fluorescent dye and a fluorescent dye, or, alternatively, a double labeled substrate according to the present invention may be constructed using two fluorescent dyes. However, it should be noted that the structure of the GFP and related protein molecules might not be able to stack and quench in the same manner as conventional dyes. As a result, where double-labeled substrates according to the present invention are constructed using one of the various GFP molecules, it may be necessary to include only one GFP molecule in the combination of two dyes covalently attached to the double-labeled substrate (See, U.S. Patents 5,958,713, 5,925,558, and 5,912,137). Nevertheless, the combination, nature and location of the two dyes included in a

double-labeled substrate of the present invention is of relatively little import, provided that the two dyes stack to form a quenched intramolecular dye dimer when the double-labeled substrate is unmodified, the two dyes dissociate upon structural modification of the double-labeled substrate, and the dissociation of the two dyes upon structural
5 modification results in a change in the double-labeled substrate's fluorescence or absorbance characteristics. Thus, the construction of a double-labeled substrate according to the present invention is variable, and, depending on the application, the double-labeled substrate of the present invention may include one or more of many biomolecular substrates and any suitable combination of two labels.

10 Also included within the scope of the present invention are methods of using the double-labeled biomolecular substrates of the present invention. For example, methods of using the double-labeled substrates herein described for the assay of protein kinase activity *in vitro* and in living cells fall within the scope of the present invention, as do methods of using the same double-labeled substrates for high-throughput
15 screening. The double-labeled substrates are also useful for diagnostic and therapeutic applications and for methods which facilitate the discovery of substrates, activators, and inhibitors for novel protein kinases. Significantly, most efforts to discover drugs affecting protein kinase activity are presently aimed at screening for possible protein kinase activators and inhibitors.

20 A preferred method according to the present invention for assaying structural modification of biomolecules *in vitro* is homogenous, comparatively simple, and includes the steps of providing a double-labeled substrate as herein described, including the double-labeled substrate in a sample, and quantifying any resultant change in fluorescence or absorbance resulting from the structural modification of the double-
25 labeled substrate. Because only double-labeled substrate which is structurally modified exhibits a change in fluorescence or absorbance, this method requires no separation of the unmodified double-labeled substrate from the modified double-labeled substrate before the modification of the double-labeled substrate can be accurately assayed. Further, the assay methods of the present invention require no special reagents other
30 than the double-labeled substrate, and the measurement of changes in fluorescence or absorbance of the double-labeled substrate can be easily achieved using a variety of well known instruments, such as, for example, known spectrometers, 96-well and 384-

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well microtiter plate readers, other multichannel readers, and micro-array instruments. Therefore, the methods of assaying covalent biomolecular modifications according to the present invention provide advantages over currently used assays in terms of simplicity, throughput, versatility, and economy.

5 Because the method already described requires no separation steps, it can be easily modified in order to assay processes of covalent biomolecular modification in living cells. A preferred method for assaying biomolecular structural modification in living cells includes providing a double-labeled substrate of the present invention, introducing the double-labeled substrate into living cells using techniques well known
10 in the art, such as microinjection, pinocytosis, or facilitated uptake, and quantifying any change in fluorescence or absorbance resulting from the structural modification of the double-labeled substrate using well known instruments, such as, for example, known spectrometers, fluorescence microscopes, plate readers, cell counters, and cell sorters. Again, this method requires no separation steps (although they may be used), and, thus,
15 allows for the continuous, real-time assay of the processes resulting in structural modification of biomolecules in living cells. Monitoring of biomolecular structural modification activities in living cells could be used for purposes of basic research, drug discovery, diagnosis of disease states, or efficacy of therapy following targeted drug treatment.

20 Also included within the scope of the invention are methods for the assay of covalent biomolecular modifications performed *in vitro* and in living cells which simultaneously monitor different processes of biomolecular structural modification by utilizing various double-labeled substrates of the present invention, each double-labeled substrate being designed to specifically assay the activity of a different process by
25 which biomolecules are modified. Such a method is similar to those already detailed, except that, in order to accurately simultaneously monitor the activity of multiple processes of covalent biomolecular modification, each of the different double-labeled substrates must be designed with unique and distinguishable spectral properties. Because they enable the simultaneous and continuous monitoring of multiple processes
30 by which biomolecules are structurally modified, such assay methods will likely hasten the discovery of exactly which processes of biomolecular structural modification are associated with specific disease states.

The double-labeled substrates of the present invention can also be used in methods facilitating the discovery of drugs which target intracellular processes of covalent biomolecular modification. Such a preferred method would include the steps of providing a sample containing the modifying enzyme(s) to be targeted, introducing
5 into the sample a drug designed to target a particular intracellular process of covalent biomolecular modification, introducing into the sample a double-labeled substrate specific for the targeted modification process, and quantifying any change in fluorescence or absorbance resulting from the structural modification of the double-labeled substrate using well known instruments. Since the covalent modification of
10 biomolecules represents one of the major mechanisms by which intracellular signaling occurs, many processes facilitating such modifications are likely to be important drug targets, and methods of drug discovery facilitated by the double-labeled peptides of the present invention are of particular importance.

Yet another aspect of the present invention is a method of using the double-labeled substrates of the present invention to identify substrates for novel modifying
15 enzymes. The human genome is estimated to contain thousands of different enzymes responsible for the intracellular modification of biomolecules, many of which are likely to be critically involved in disease processes. Protein kinases represent but one superfamily of such enzymes, yet there are currently hundreds of putative protein
20 kinases in sequence databases, such as GenBank and "EST" databases, whose function and regulation are entirely unknown. These putative protein kinases can be identified by their homology to known protein kinases and can be cloned and expressed as proteins, but their enzymatic properties cannot be studied without an appropriate peptide or protein substrate. A preferred embodiment of a method of identifying
25 peptide substrates for these protein kinases or other putative enzymes with unknown enzymatic properties involves constructing (*e.g.*, synthesizing) combinatorial libraries of double-labeled substrates according to the present invention with core molecular backbones constructed with randomized amino acid (in the case of peptides) and nucleotide (in the case of DNA and RNA, etc.) sequences, systematically introducing
30 individual double-labeled substrates from the combinatorial libraries into a sample containing the novel protein kinase or other enzyme of unknown activity, and quantifying any change in substrate fluorescence or absorbance which results from

covalent modification of the double-labeled substrate. Structural modification of specific double-labeled substrates in the combinatorial library by the novel enzyme of interest would result in a change in their spectral properties, which would permit these biomolecules to be identified by standard methods. Once substrates are identified for a novel enzyme, they can be used to characterize the activity and properties of the enzyme *in vitro* and in living cells. Further, such substrates would also facilitate drug development for the novel enzyme of interest using the methods described herein (*e.g.*, to identify specific inhibitors or activators of a newly discovered protein kinase).

The present invention is further directed to kits which utilize the double-labeled substrates and methods described herein to detect and/or quantify covalent biomolecular modification. A preferred embodiment of such a kit would include a container, one or more different double-labeled substrates of the present invention contained within the container, and instructions for use. The kits may also include, for convenience, buffers and other reagents necessary to carry out the assay, and samples of enzyme for calibration purposes. The reagents included with the kits can be varied depending on the application and in order to optimize the sensitivity of the assay.

A further aspect of the invention is the use of double-labeled substrates to detect protein kinase activities and other modification reactions in living cells. A preferred embodiment of a method of detecting protein kinase activity in living cells involves constructing double-labeled protein kinase substrates as probes which are sufficiently cell permeable, or capable of cell permeability with inducement measures well known to one skilled in the art, and according to the present invention, with core molecular backbones constructed of various peptide sequences. In this situation, the protein kinase activity would result in a covalent structural modification of the double-labeled substrate, leading to a change in fluorescence or absorbance and *in situ* detection of kinase activity using instruments well known in the art, such as, for example, known spectrometers, fluorescence microscopes, plate readers, cell counters, and cell sorters.

The invention is further described with the aid of the following illustrative example.

Example I

A double-labeled protein kinase substrate can be designed, synthesized, characterized, and used to assay the activity of PKA and other protein kinases.

The core molecular backbone of the substrate is the synthetic peptide sequence
5 Asp-Ser-Gln-Arg-Arg-Arg-Glu-Ile-Leu-Ser-Arg-Arg-Pro-Ser-Tyr-Arg-Arg-Ile-Leu-
Asn-Asp-Leu-Cys-Gly (SEQ. ID. NO.: 5). This synthetic peptide sequence is based on
the native sequence for KID, which is Arg-Arg-Pro-Ser-Tyr-Arg-Lys-Ile-Leu-Asn-Asp-
Leu (SEQ. ID. NO.: 6). To arrive at the peptide sequence of the core molecular
backbone, the Lys residue of the native KID sequence was replaced by an Arg residue
10 to facilitate site specific labeling of the peptide's α -amino group. Replacing the Lys
residue of the native KID sequence resulted in the synthetic KID sequence Arg-Arg-
Pro-Ser-Tyr-Arg-Arg-Ile-Leu-Asn-Asp-Leu (SEQ. ID. NO.: 7), which represents the
sequence generally referred to herein as "the KID sequence." Next, a Cys residue was
added to the C-terminus of the synthetic KID sequence to allow labeling of the
15 molecular backbone with a dye through the sulfhydryl group in the cysteine residue, and
a Gly residue was added at the terminal Cys residue to facilitate peptide synthesis.
Finally, the additional peptide sequence Asp-Ser-Gln-Arg-Arg-Arg-Glu-Ile-Leu-Ser
(SEQ. ID. NO.: 8) was added at the Arg residue of the N-terminus of the KID sequence
to give the final peptide more helical structure.

20 The synthetic peptide sequence of the core molecular backbone of the substrate
was synthesized on a benzhydrylamine resin using conventional (tBOC) solid phase
peptide synthetic chemistry. See, e.g. Barany and Merrifield in *The Peptides, Analysis,
Synthesis, Biology*, Vol. 2, E. Gross and J. Meienhofer, eds., (Acad. Press, New York,
1980), Glass, D.B., *Methods Enzymol.*, 99, 119-139 (1983). After synthesis, the peptide
25 sequence was cleaved from the resin with anhydrous HF using standard protocols which
yield a crude side-chain deprotected peptide with an amide C-terminus, and the
synthetic peptide sequence was purified to homogeneity by HPLC using a C4 reverse-
phase column. The mass of the purified peptide sequence was confirmed by mass
spectrometry. The synthetic polypeptide sequence of SEQ. ID. NO.: 5 was selected as
30 an exemplary core molecular backbone for a double-labeled substrate of the present
invention not only because the KID sequence embedded in the synthetic polypeptide is
known to contain the specificity determinants of several protein kinases, including

PKA, but also because this exemplary synthetic peptide sequence has proven to undergo a phosphorylation-dependent change in conformation.

After purification, the core molecular backbone was conjugated with two dyes to form a double-labeled substrate. First, the synthetic peptide sequence of the core molecular backbone was conjugated with tetramethylrhodamine-5-maleimide. The maleimide on the dye reacts with the cysteine residue at the C-terminus in the KID region, and the maleimide group serves as the link between the sulfhydryl group on the cysteine and the rhodamine group. The single-labeled substrate was then conjugated at the N-terminus with either 5-carboxyfluorescein, succinimidyl ester or 5-carboxytetramethylrhodamine, succinimidyl ester. The succinimidyl ester group reacts with the amino group at the N-terminus of each peptide to form a carboxamide bond with the dye. Following HPLC purification on a C4 reversed-phase column, the double-labeled substrate was subjected to analysis by mass spectrometry analysis, UV absorbance spectrophotometry, and fluorescence spectrophotometry.

After such analyses, the double-labeled substrate was phosphorylated with PKA. The phosphate acceptor amino acid in this double-labeled substrate is the serine residue found within the embedded KID sequence.

As illustrated in FIG. 5 through FIG. 9, phosphorylation of double-labeled substrates prepared as herein described results in detectable changes in the absorbance and fluorescence characteristics of the dyes included in the double-labeled substrates.

FIG. 5. illustrates the absorbance peaks for fluorescein and rhodamine before and after phosphorylation of a double-labeled substrate having a fluorescein dye and a rhodamine dye molecule conjugated thereto. The unphosphorylated substrate exhibits an absorbance maximum for fluorescein at 500nm and an absorbance maximum for rhodamine at 552 nm. However, after phosphorylation, the two absorbance peaks shift to 498 nm and 548 nm for fluorescein and rhodamine, respectively.

FIG. 6 and 7 illustrate the even more dramatic phosphorylation dependent changes in the fluorescence characteristics a double-labeled substrate having a fluorescein and a rhodamine molecule conjugated thereto. As can be seen in FIG. 6, the fluorescence of the fluorescein label increased 340% after phosphorylation, and FIG. 7 illustrates that phosphorylation of the double-labeled substrate caused a 35% increase in the fluorescence of the rhodamine label.

As mentioned, the molecular backbone described herein may also be labeled with, among other combinations, two rhodamine dyes instead of a rhodamine dye and a fluorescein dye, and FIG. 8 and FIG. 9 illustrate the phosphorylation-dependent changes in the optical properties of such a double-labeled substrate. As can be seen in FIG. 8, in its unphosphorylated state, the double-labeled substrate exhibited two absorbance maxima. The larger peak is at 520 nm, while the smaller peak is at 552 nm. After phosphorylation, the peak at 520 nm decreases in size while the peak at 552 nm shifts to 550 nm and increases in size. Moreover, as can be appreciated from FIG. 9, the fluorescence of rhodamine increases 69% after phosphorylation.

The results illustrated in FIG. 5 through FIG. 9, therefore, show that the fluorescence of at least one label conjugated to the double-labeled substrates is quenched when the substrates are found in their unphosphorylated state. These results indicate that when the double-labeled substrates are not phosphorylated, the two dyes included in each double-labeled substrate stack on each other to form an intramolecular dye dimer, resulting in the reduction of the fluorescence of at least one of the dyes included in the dye dimers. Stacking is also indicated by the observation that the UV absorbance spectrum of the unphosphorylated double-labeled substrate differs markedly from the spectra of the same substrate after phosphorylation.

As is also apparent by reference to results illustrated in FIG. 5 through FIG. 9, phosphorylation of the double-labeled substrates results in an increase in the intensity of the fluorescent emission peak of at least one dye conjugated to the double-labeled substrates. This indicates that phosphorylation of the double-labeled substrate causes a dissociation of the intramolecular dimer. In each instance, there was a large increase in fluorescence intensity of at least one of the dyes conjugated to the double-labeled substrate, thereby providing a high signal-to-noise ratio. Sensitivity is also excellent with changes in dye emission intensity being observable at low nanomolar concentrations of peptide in a standard spectrofluorometer. The favorable sensitivity and signal-to-noise ratio indicate the double-labeled substrate will be useful for monitoring protein kinase activity in a variety of applications.

The procedures and methods described herein can be employed to prepare and use double-labeled protein kinase substrates for assaying most any other protein kinase. For example, the KID sequence included in the core molecular backbone described

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herein may be modified to contain an appropriate consensus sequence for a given protein kinase determinant. Such consensus sequences can be found in the literature for many common kinases such as PKA, PKC, CaM kinase II, etc. (*cf.*, Songyang et al. Current Biol. 4:479, 1994). Moreover, the double-labeled substrate of the present invention can be prepared for assaying most any other intracellular processes leading to the structural modification of protein or other biomolecules. Thus, even though the present invention has been herein described in terms of certain preferred embodiments and specific examples, such descriptions are illustrative only and do not limit the scope of the present invention. The scope of the present invention is to be defined by the appended claims.

CLAIMS

What is claimed is:

1. A biomolecular substrate comprising:
a core molecular backbone;
5 a first fluorescent dye associated with said core molecular backbone; and
a second dye associated with said core molecular backbone which, when said
biomolecular substrate is not covalently modified, associates with said first fluorescent
dye forming a quenched intramolecular dye dimer, but which, when said biomolecular
substrate is covalently modified, dissociates from said first fluorescent dye .
10
2. The biomolecular substrate of claim 1, wherein said second dye is a
fluorescent dye.
3. The biomolecular substrate of claim 1 further comprising a first spacer
15 segment included at a first terminus of said core molecular backbone.
4. The biomolecular substrate of claim 1 further comprising a first spacer
segment included at a first terminus of said core molecular backbone and a second
spacer segment included at a second terminus of said core molecular backbone.
20
5. The biomolecular substrate of claim 1, wherein said first fluorescent dye
is selected from a group consisting of fluorescein, rhodamine, cyanine, Oregon Green,
Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin,
naphthalene, pyridyloxazole, anthracene, fluorescamine, acridine, benzofuran,
25 anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole,
carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimeane, phenanthroline, Yellow
Fluorescent Protein, and Green Fluorescent Protein.
6. The biomolecular substrate of claim 1, wherein said second dye is
30 selected from a group consisting of fluorescein, rhodamine, cyanine, Oregon Green,
Texas Red, Lucifer Yellow, BODIPY, rhodol, coumarin, pyrene, eosin, erythrosin,
naphthalene, pyridyloxazole, anthracene, fluorescamine, acridine, benzofuran.

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anthranilic acid, aminobenzoic acid, N-methylisatoic acid, isoluminol, bezoxadiazole, carboxybenzoyl-quinoline-carboxyaldehyde, salicylate, bimeane, phenanthroline, Yellow Fluorescent Protein, and Green Fluorescent Protein.

5 7. The biomolecular substrate of claim 1, wherein said core molecular backbone comprises a molecule selected from a group consisting of a peptide, a protein, a nucleic acid, a sugar, a lipid, a receptor, and a biopolymer.

 8. The biomolecular substrate of claim 1, wherein said core molecular
10 backbone comprises an amino acid sequence.

 9. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a substrate determinant.

15 10. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a protein kinase substrate.

 11. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a nucleotide sequence.

20 12. The biomolecular substrate of claim 1, wherein said core molecular backbone includes a lipid.

 13. The biomolecular substrate of claim 1, wherein said core molecular
25 backbone includes a biopolymer comprising a covalent combination of molecules selected from the group consisting of amino acids, nucleic acids, sugars, and lipids.

 14. The protein kinase substrate of claim 1, wherein the core molecular backbone comprises a KID peptide sequence, the first fluorescent dye comprises
30 fluorescein-succinimidyl ester, and the second dye comprises tetramethylrhodamine-maleimide.

15. A method of assaying covalent biomolecular modification in a sample comprising:

providing the sample with a biomolecular substrate comprising:

- a biomolecular substrate;
- 5 a core molecular backbone;
- a first fluorescent dye associated with said core molecular backbone; and
- a second dye associated with said core molecular backbone which, when said biomolecular substrate is not covalently modified, associates with said first fluorescent dye forming a quenched intramolecular dye dimer and affecting
- 10 fluorescence or absorbance characteristics of said biomolecular substrate, but which dissociates from said first fluorescent dye when said biomolecular substrate is covalently modified;
- introducing said biomolecular substrate to said sample; and
- quantifying a resultant change in said fluorescence or absorbance characteristics
- 15 of said biomolecular substrate.

16. The method of claim 15, wherein said biomolecular substrate is introduced into said living cells.

20 17. The method of claim 15, wherein said sample includes a drug targeting a specific process of covalent biomolecular modification.

18. The method of claim 16, wherein said sample includes a drug targeting a specific process of covalent biomolecular modification.

25

19. The method of claims 15, wherein said step of providing a biomolecular substrate comprises providing two or more different biomolecular substrates, each of said two or more biomolecular substrates being specific for different processes of covalent biomolecular modification and having unique and distinguishable spectral

30 properties.

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20. The method of claim 16, wherein said step of providing a biomolecular substrate comprises providing two or more different biomolecular substrates, each of said two or more biomolecular substrates being specific for different processes of covalent biomolecular modification and having unique and distinguishable spectral properties.

21. The method of claim 15, wherein the step of quantifying the resultant change in said fluorescence or absorbance characteristics of said biomolecular substrate comprises quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate without separating biomolecular substrate which has been covalently modified from biomolecular substrate which has not been covalently modified.

22. The method of claim 16, wherein the step of quantifying the resultant change in fluorescence or absorbance characteristics of said biomolecular substrate comprises quantifying the resultant change in fluorescence or absorbance of said biomolecular substrate without separating biomolecular substrate which has been covalently modified from biomolecular substrate which has not been covalently modified.

20

23. A method of assaying protein kinase activity comprising:
providing a biomolecular substrate comprising:

a KID peptide sequence;

a molecule of fluorescein; and

25 a molecule of tetramethylrhodamine which, when said biomolecular substrate is not phosphorylated, associates with said molecule of fluorescein forming an intramolecular dye dimer, but which dissociates from said molecule of fluorescein when said biomolecular substrate is phosphorylated by a protein kinase;
30 providing a sample;
introducing said protein kinase substrate to said sample; and

quantifying a resultant change in fluorescence or absorbance of said biomolecular substrate.

24. The method of claim 23, wherein the step of quantifying the resultant
5 change in fluorescence or absorbance of said biomolecular substrate comprises
quantifying the resultant change in fluorescence or absorbance of said biomolecular
substrate without separating biomolecular substrate which has been phosphorylated
from biomolecular substrate which has not been phosphorylated.

10 25. A method of identifying substrates of novel enzymes which catalyze
covalent structural modifications of particular proteins or peptide sequences
comprising:

gathering a combinatorial library of unique double-labeled substrates, said
unique double-labeled substrates each comprising:

15 a particular, randomized core amino acid sequence;

a first fluorescent dye associated with said particular, randomized core
amino acid sequence; and

a second dye associated with said particular, randomized core amino
acid sequence which, when said unique double-labeled substrates are not
20 covalently modified, associates with said first fluorescent dye forming a
quenched intramolecular dye dimer and affecting the fluorescence or absorbance
characteristics of said unique double-labeled substrates, but which dissociates
from said first fluorescent dye when said unique double-labeled substrates are
covalently modified;

25 systematically contacting each of said unique double-labeled substrates with a
novel enzyme;

quantifying any change in fluorescence or absorbance characteristics of each of
said unique double-labeled substrates;

30 selecting members of the library undergoing a fluorescence change or an
absorbance change; and

determining the amino acid sequence of said selected members of the library.

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26. A kit comprising:
a container;
one or more different biomolecular substrates contained within said container,
each of said one or more different biomolecular substrates comprising:
5 a core molecular backbone;
a first fluorescent dye associated with said core molecular backbone;
a second dye associated with said core molecular backbone which, when
said
biomolecular substrate is not covalently modified, associates with said first fluorescent
10 dye forming an intramolecular dye dimer, but which dissociates from said first
fluorescent dye when said biomolecular substrate is catalytically or non-catalytically
covalently modified; and
a sample of enzyme standard with which to standardize the assay.
- 15 27. A method of identifying substrates of novel enzymes which catalyze
covalent structural modifications of particular nucleic acids comprising:
gathering a combinatorial library of unique double-labeled substrates, said
unique double-labeled substrates each comprising:
a particular, randomized core nucleic acid sequence;
20 a first fluorescent dye associated with said particular, randomized core
nucleic acid sequence; and
a second dye associated with said particular, randomized core nucleic
acid sequence which, when said unique double-labeled substrates are not
covalently modified, associates with said first fluorescent dye forming a
25 quenched intramolecular dye dimer and affecting the fluorescence or absorbance
characteristics of said unique double-labeled substrates, but which dissociates
from said first fluorescent dye when said unique double-labeled substrates are
covalently modified;
systematically contacting each of said unique double-labeled substrates with a
30 novel enzyme;
quantifying any change in fluorescence or absorbance of each of said unique
double-labeled substrates;

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selecting members of the library undergoing a fluorescence or absorbance change; and
determining the nucleotide sequence of said selected members of the library.

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FIG. 1

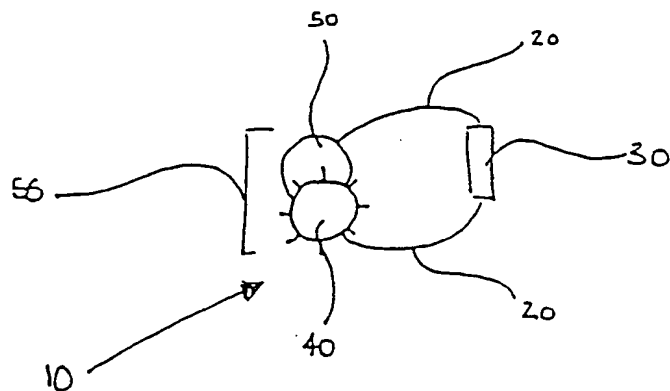


FIG. 2

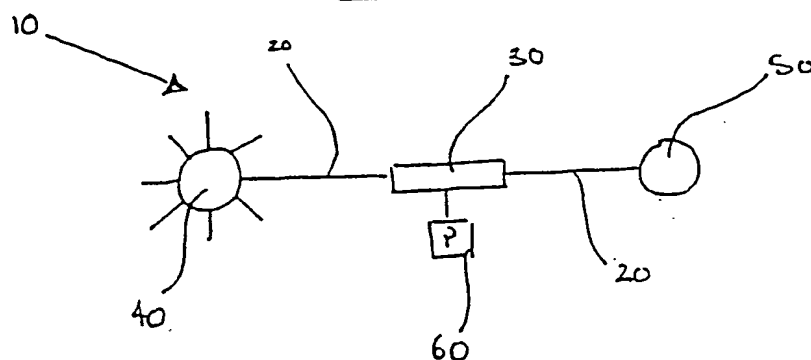
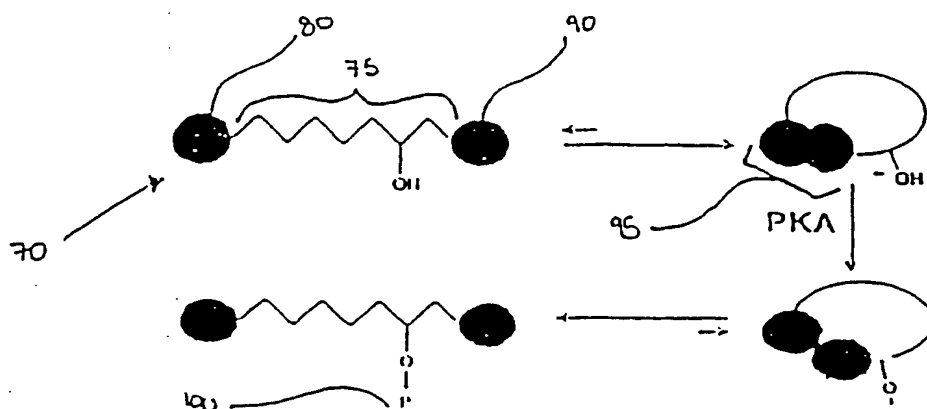
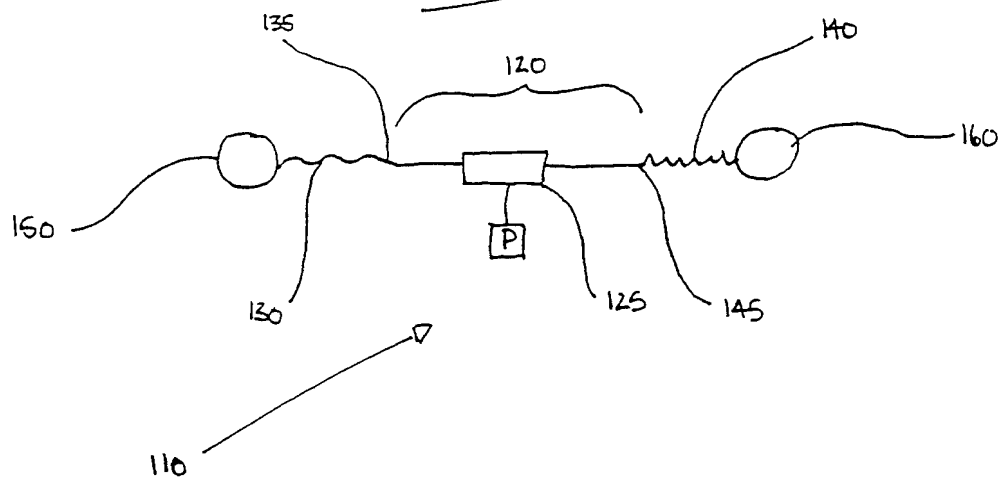
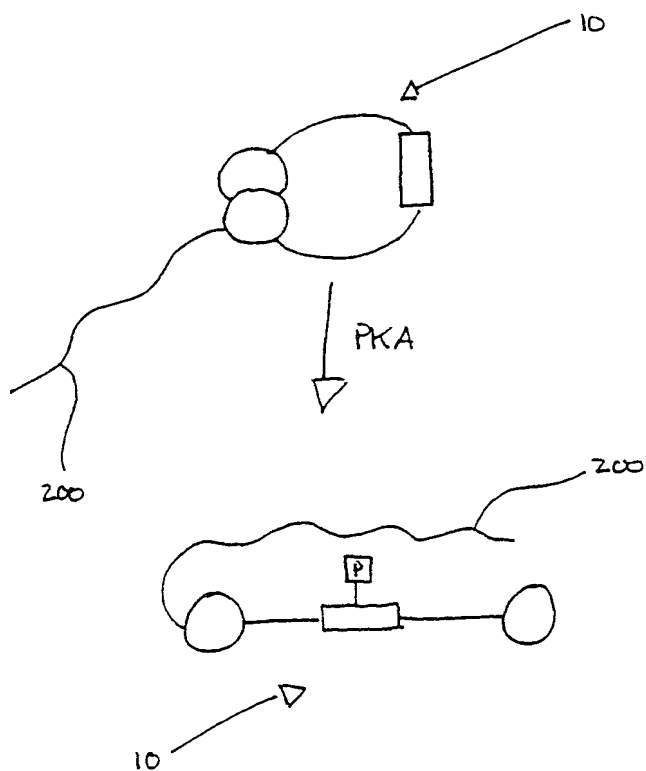


FIG. 3



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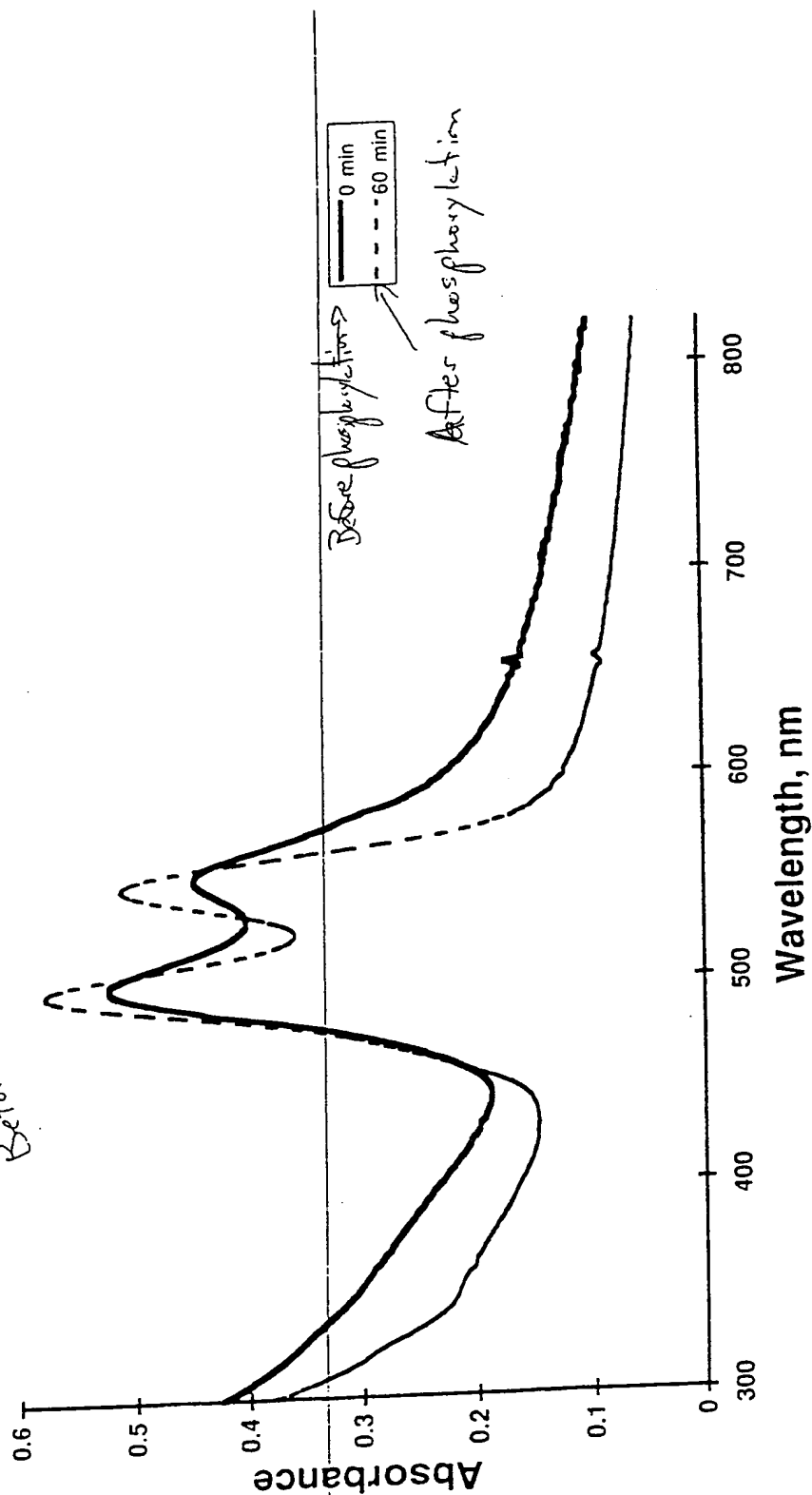
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FIG. 4FIG. 10

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Absorbance Chart 1

Absorbance Spectrum of F-DSQRRREILS-KID-R After PKA Phosphorylation

Before andFIG. 5

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Ex 480 nm Chart 1

Before and After
F-DSQRRREILS-KID-R Fluorescence
 PKA Phosphorylation, Ex 480 nm (Fluorescein)

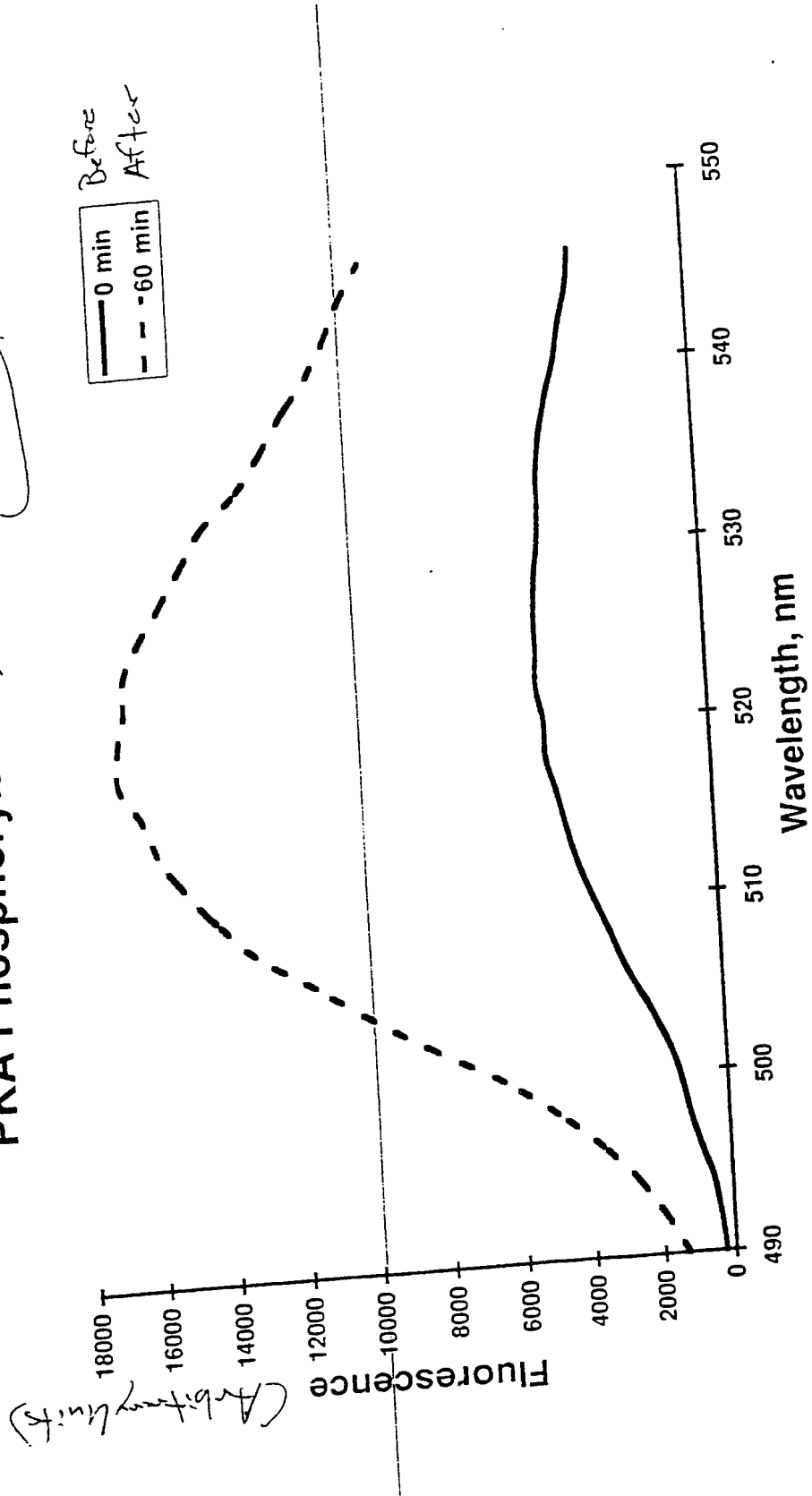
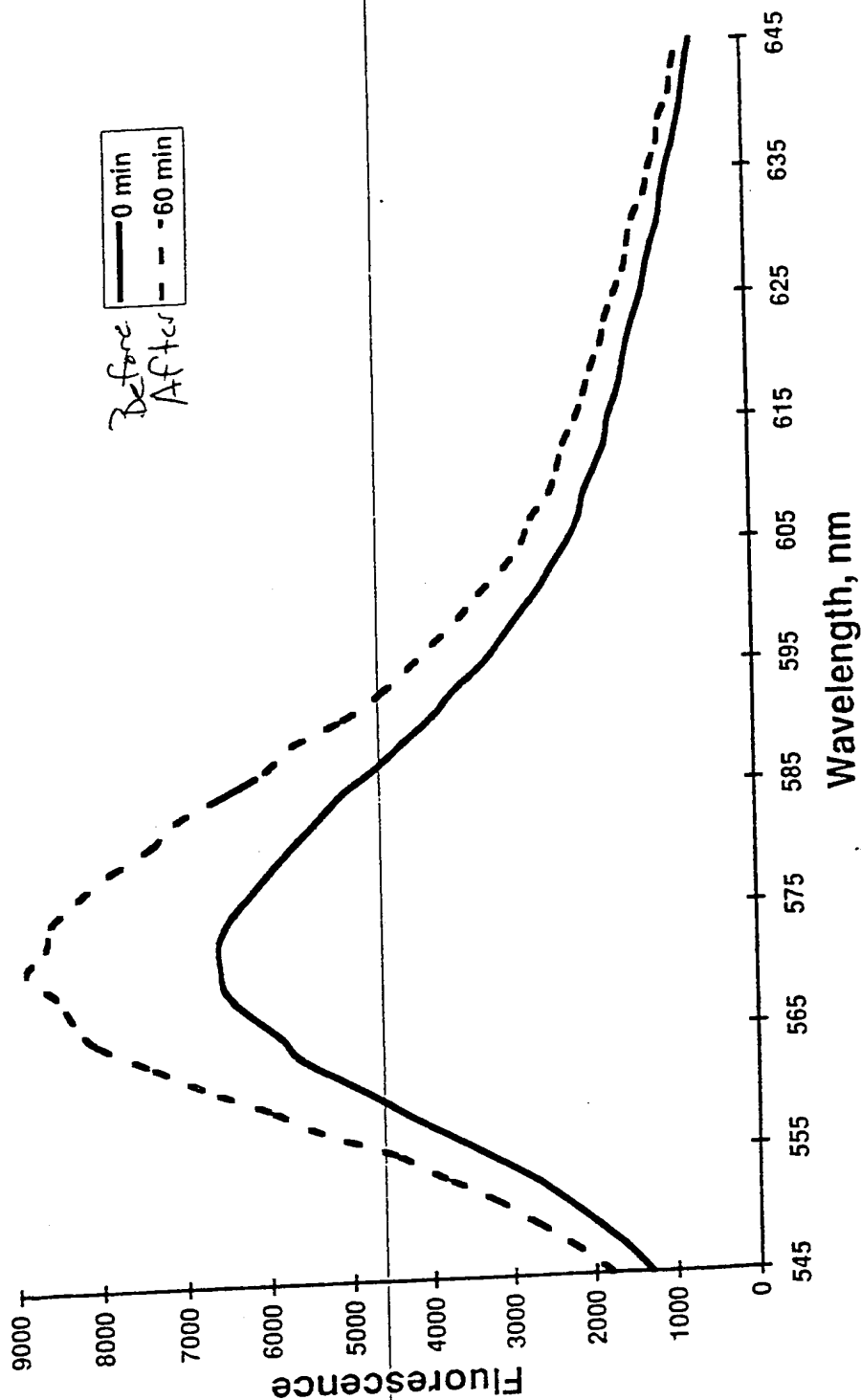


FIG. 6

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Before and After
F-DSQRRREILS-KID-R Fluorescence/After PKA
Phosphorylation, Ex 535 nm \rightarrow (Rhodamine)

FIG. 7

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Fig. 4

Absorbance Spectrum of TMR-DSQRRREILS- KID-TMR After PKA Phosphorylation

Before and

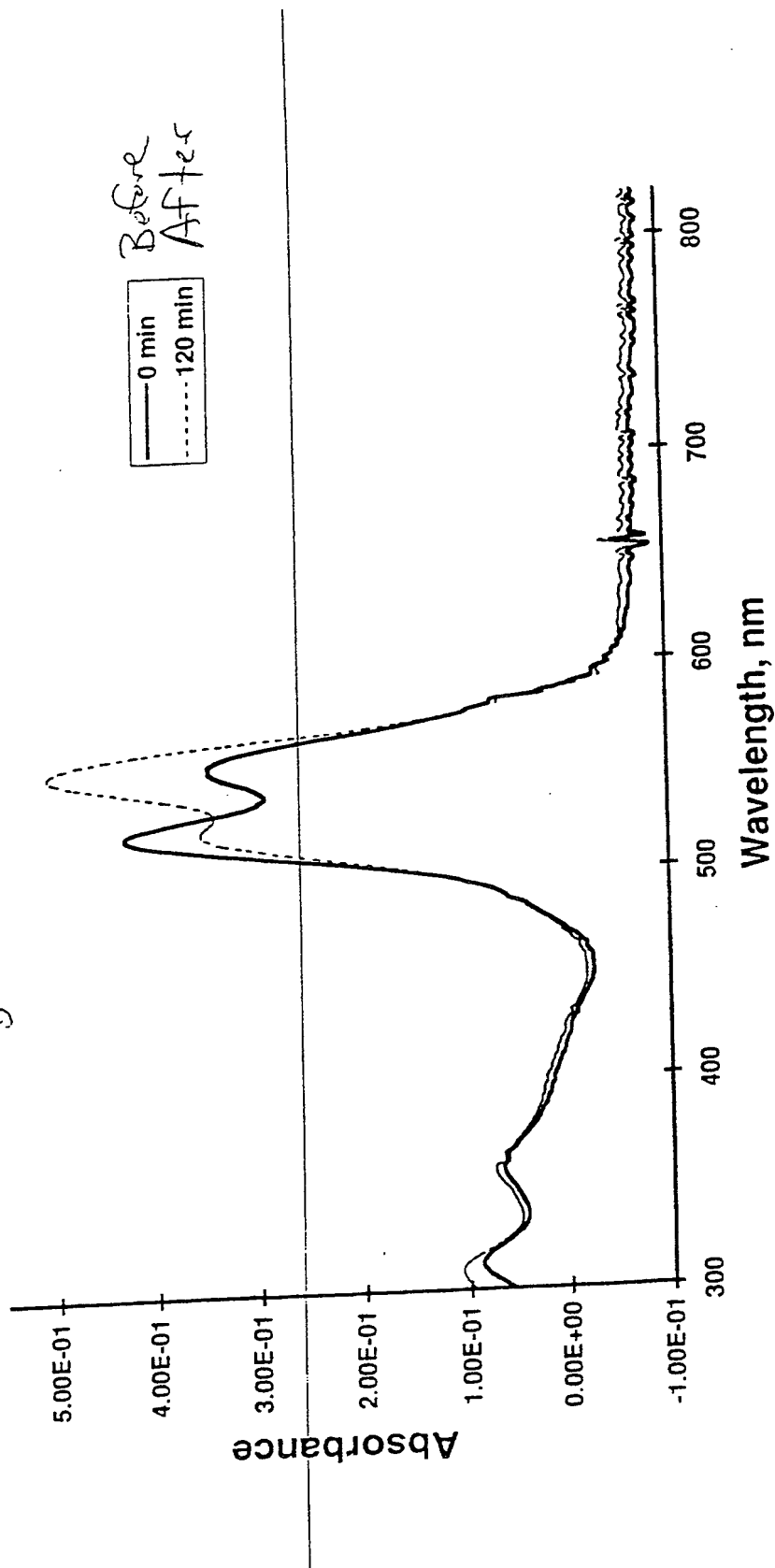


Fig. 8

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Fluorescence of TMR-DSQRRREILS-KID-TMR *Before and*
After PKA Phosphorylation, Ex 545 nm

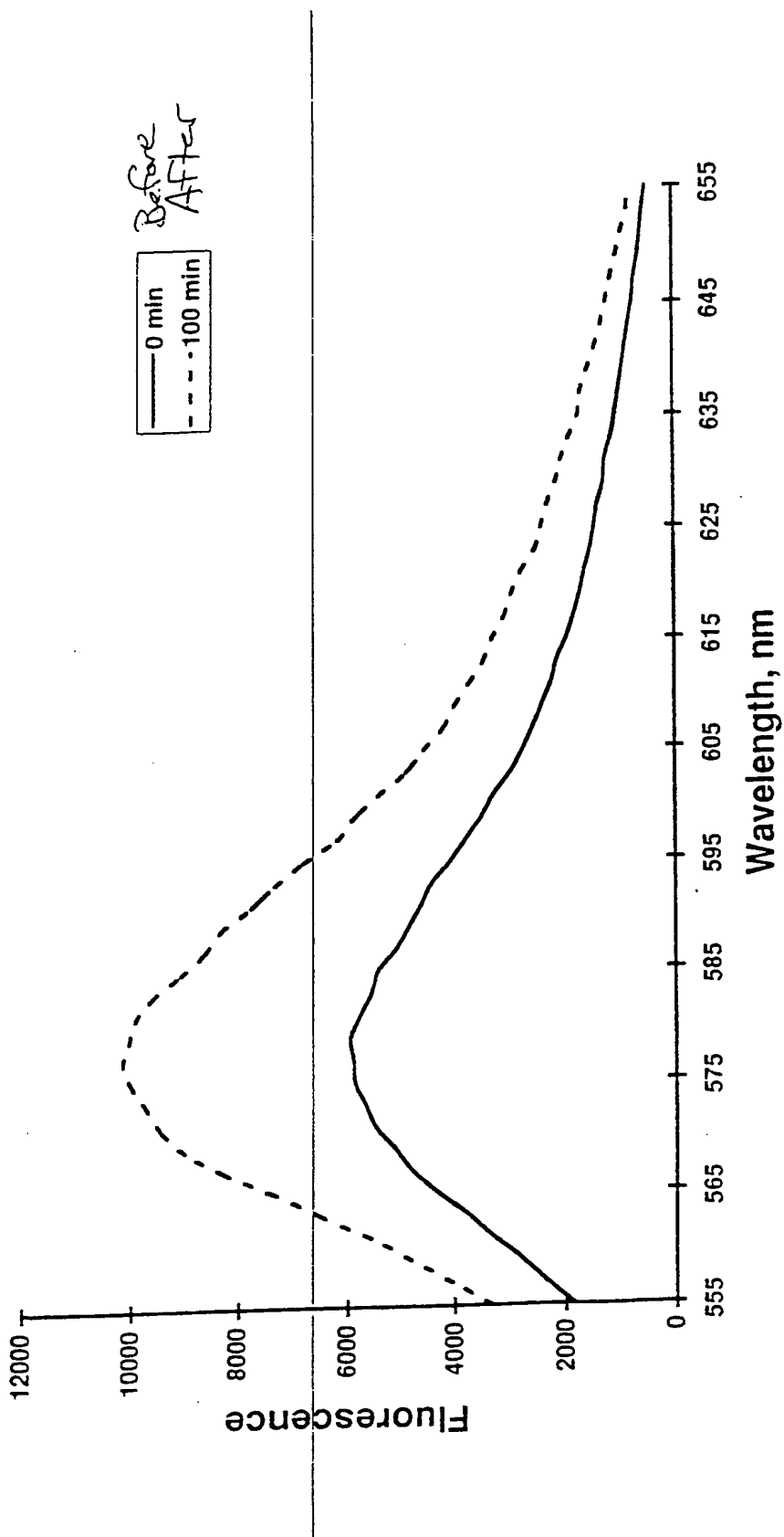


FIG. 9

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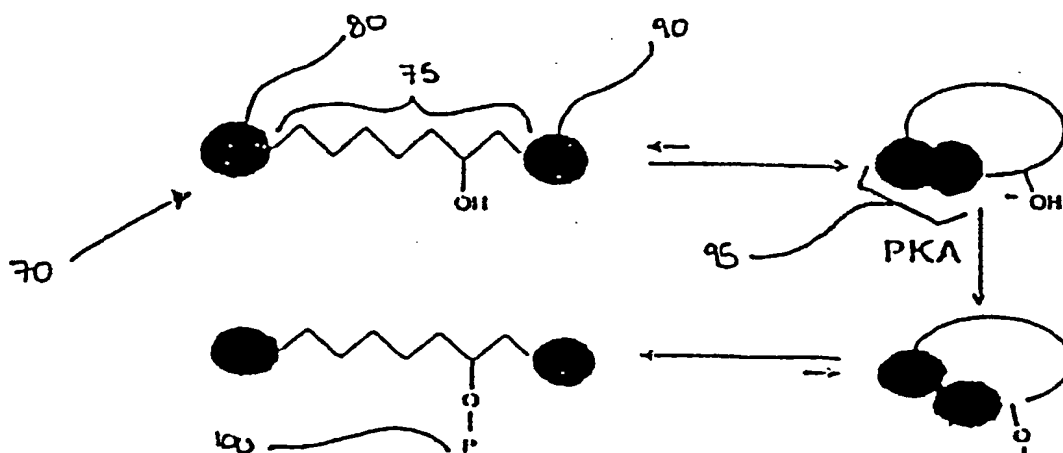
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(54) Title: HOMOGENEOUS FLUORESCENCE METHOD FOR ASSAYING STRUCTURAL MODIFICATIONS OF BIOMOLECULES



(57) Abstract: Double-labeled protein biomolecular substrates (70) and methods for the homogenous assay of processes which include covalent modification of the substrates (70) to form a detectable species are described. The biomolecular substrates (70) of the instant invention are labeled at two positions (80, 90) with two fluorescent dyes or with a fluorescent dye and a nonfluorescent dye. The two labeling dyes of the unmodified substrate (70) stack (95), thereby quenching the substrate's fluorescence. Upon covalent modification of the double-labeled substrate (70), however, the intramolecularly stacked dyes (95) dissociate and the fluorescence changes markedly. Examples are described for the preparation and use of substrates (70) for phosphorylation assays. Methods of invention do not require separation of the modified and unmodified substrates (70), nor do they require other special reagents or radioactive materials. Therefore the substrates can be used for monitoring intracellular processes of living cells.

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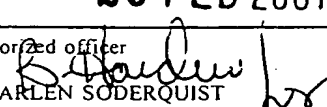
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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/40495

A. CLASSIFICATION OF SUBJECT MATTER IPC(7) :G01N 33/53 US CL :435/4, 7.72, 15.21; 436/86, 89, 172 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/4, 7.72, 15.21; 436/86, 89, 172 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Please See Extra Sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	K. B. Lee et al, "A New Approach to Assay Endo-Type Carbohydrases: Bifluorescent-Labeled Substrates for Glycoamidases and Ceramide Glycanases" Analytical Biochemistry, 01 September 1995, Vol. 230, No. 1, pages 31-36, see entire document.	1-2,5-7,13, 15,21 ----- 3-4,8-12,14,16-20,22-27
X - Y	K. Matsuoka et al, "A Bi-Fluorescence-Labeled Substrate for Ceramide Glycanase Based on Fluorescence Energy Transfer" Carbohydrate Research, 16 October 1995, Vol. 276, No. 1, pages 31-42, see entire document.	1,2,5-7,13,15,21 ----- 3,4,8-12,14,16-20,22-27
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family
Date of the actual completion of the international search 16 JANUARY 2001		Date of mailing of the international search report 23 FEB 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer  ARLEN SODERQUIST Telephone No. (703) 308-3989

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/40495

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	G. Harnischfeger "Effect of Chemical Modification of Amino Groups by Fluorescamine on Partial Reactions of Photosynthesis" <i>Biochimica et Biophysica Acta</i> , 7 September 1978, Vol. 503, No. 3, pages 473-479, see entire document.	1-27
Y	US 4,777,128 A (LIPPA) 11 October 1988, see entire document.	1-27
Y	Z. Zhang et al, "Amylase Substrate Based on Fluorescence Energy Transfer" <i>Analytica Chimica Acta</i> , 17 September 1990, Vol. 236, No. 2, pages 251-256, see entire document.	1-27
Y	A.-P. Wei et al, "Antibody-Mediated Fluorescence Enhancement Based on Shifting the Intramolecular Dimer/Monomer Equilibrium of Fluorescent Dyes" <i>Analytical Chemistry</i> , 01 May 1994, Vol. 66, No. 9, pages 1500-1506, see entire document.	1-27
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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	D. A. Pearce et al, "Peptidyl Chemosensors Incorporating a FRET Mechanism for Detection of Ni(II)" Bioorganic & Medicinal Chemistry Letters, 04 August 1998, Vol. 8, No. 15, pages 1963-1968, see entire document.	1-27
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B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

CA, BIOSIS, and MEDLINE files in STN

search terms: kinase, fluoresc?, label?, absor?, resonant?, energy, transfer?, Exchang?, covalent?, double, di. bi. two, label, fluoroph?, modif?, bind?, bond?, complex?, bound?, immobil?

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